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Optimization of Process Variables for the Preparation of Gellan Gum-Raloxifene Nanoparticles Using Statistical Design

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ABSTRACT

Raloxifene loaded gellan gum nanoparticles were prepared using emulsion cross linking method. A central composite design was applied to optimize the concentration of gellan gum, di-octyl sodium sulfosuccinate (AOT) and raloxifene to obtain an optimum formulation of gellan gum-raloxifene nanoparticles. The percentage of encapsulation, particle size and zetapotential were taken as response variables. A second order polynomial equation obtained from the regression analysis of the data was used to find the optimum level of the variables. The optimum concentrations were found to be gellan gum 1.31%, w/v, AOT 9.88%, w/v, raloxifene HCl (Drug) 1.08%, w/v. The optimized nanoparticle preparation showed 97% encapsulation, 256 nm particle size and zetapotential of -49 mV.

Keywords: Polymer Nanoparticles, Biopolymer, Gellan Gum, *Sphingomonas Paucimobilis,* Drug Delivery, Raloxifene HCl.

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INTRODUCTION

Gellan gum is an anionic heteropolysaccharide produced by *Sphingomonas paucimobilis* under aerobic fermentation[1]. Gellan have wide applications in food and pharmaceutical industries as thickening, binding and emulsifying agent. Gellan is used as a vehicle for ophthalmic preparations[1,2] and other sustained release pharmaceutical preparations *viz*. coated tablets & capsules[4,5], implants[6], microspheres[7], beads[8], and cross linked hydrogels for tissue engineering scaffols[9]. Gellan forms firm, hard, and brittle gels, with gelation being dependent on the type of cation, ionic strength, temperature, and polymer concentration[10]. Gellan gum has been studied extensively for its use in several pharmaceutical dosage forms but studies related to the application of gellan in nanoparticle systems are very limited. Biodegradable polymeric nanoparticles exhibit various advantages for cancer therapy as they have been proved to lower systemic toxicity by providing a protective housing for the drugs which limits its interaction with the healthy cells[11-14]. They also increase drug efficacy, bioavailability, retention time, specificity, tolerability, therapeutic index and intracellular penetration of the encapsulated drugs[15]. Other potential advantages of polymeric nanoparticles include prolonged bioactivity, patient compliance due to reduction in administration frequency, protection of premature degradation of the drug, improved absorption into a selected tissue and the ability to code liver multiple drugs with synergistic effects to a particular tissue[15-17].

Raloxifene HCl has poor water solubility and is classified by Biopharmaceutics Classification System (BCS) under Class II drugs[18]. It has very low bioavailability of 2% due to low aqueous solubility and extensive first pass metabolism[19]. Raloxifene is a selective estrogen receptor (ER) modulator that acts as an estrogen agonist in bone and serum lipids; acts as an antagonist at estrogen receptors in breast and uteri. It has been recommended for use to reduce the prevalence of ER-positive breast cancer and for the treatment of postmenopausal osteoporosis[20]. Raloxifene was shown to inhibit the proliferation of breast cancer cell line *MCF-7* [21], inhibit the growth of *RT4* urothelial carcinoma cells through ER-dependent induction of apoptosis indicative of its possible use in the treatment of bladder cancer[22], prevent capase-3-dependent apoptosis in human chondrocytes[23], induce apoptosis in multiple myeloma cells[24].

The nanoparticle based delivery systems for raloxifene are limited with few previous reports about solid lipid nanoparticles[25], raloxifene nanoparticles prepared using rapid expansion of supercritical system[26], raloxifene loaded solid dispersion nanoparticles[27] and raloxifene loaded nanoparticles using synthetic aliphatic polyesters[28] to improve the solubility of raloxifene. We

have recently reported the preparation of gellan gum-raloxifene nanoparticles using emulsion cross linking method (Prakash et al., 2014).

In the present study we report the optimization of formulation variables namely concentration of gellan gum and AOT along with the percentage of drug loading to get a better gellan gum-raloxifene nanoparticles system. Three independent variables of concentration of gellan, di-octyl sodium sulfosuccinate and drug were studied for their influence on dependent variables particle size, zeta potential and % encapsulation. Multiple response simultaneous optimizations using the desirability function were then used to find experimental conditions in which the system shows the most adequate results.

MATERIAL AND METHODS

Materials

Gellan gum was produced by a recombinant *Sphingomonas paucimobilis* strain developed in our laboratory. Di-octyl sodium sulfosuccinate (AOT), methylene chloride, isopropyl alcohol and mannitol were purchased from Sigma-Aldrich. Raloxifene hydrochloride was obtained from Orchid chemical and pharmaceutical Ltd, Chennai, India as gift sample. All other chemicals used were of reagent grade.

Methods

Production, isolation and purification of gellan gum

Gellan gum was produced by recombinant *Sphingomonas paucimobilis* in a 5L fermentor (Solaris, Italy) with a working volume of 3L and the conditions were maintained as described in our previous work[1]. Gellan gum was recovered using the method described previously[30] with modification. The fermented broth was diluted ten-fold with distilled water to reduce viscosity, heated for 15 minutes and cooled then pH was adjusted to 10 followed by heating in a constant water bath for 10 minutes. The heated broth was cooled; pH was brought down to 7 and centrifuged at 10000 rpm for 1 hour to separate the cells. Gellan was recovered from supernatant heated to 95°C by precipitation with three volumes of isopropanol. The precipitate was then washed with large volume of isopropanol and freeze dried; the above steps were repeated two times to remove fermentation residues. Dried gellan was dissolved in deionized water (1%, w/v), 1% KCl and the solution was heated to 95°C. The polymer was recovered by precipitation with three volumes of isopropanol followed by centrifugation at 10000 rpm for 1 hour and freeze dried.

Experimental design

A central composite design was applied to determine the optimum concentration of three significant variables selected as formulation variables on the basis of previous trials studied. The effect of variables concentration of the polymer (gellan gum) (X_1), amount of AOT (X_2) and percentage of drug loading (X_3) on gellan gum nanoparticles formulation was studied at three experimental levels (i.e.−1, 0, +1). The central point (0, 0, 0) was studied in sextet. The experimental level for these variables was selected from our preliminary work, which indicated that an optimum could be found within the level of parameter studied. Encapsulation efficiency (Y_1), particle size (Y_2) and zeta potential (Y_3) were taken as response variables. The levels of factors used for experimental design are given in table 1. The actual level of each factor was calculated by the following equation[31]:

Table 1: Levels of factors chosen for central composite design.

		Levels Low	high
Factor	**Name**	**-1**	**+1**
X_1	Gellan gum (%, w/v)	0.1	1.0
X_2	AOT (%, w/v)	1.0	10.0
X_3	Raloxifene HCl (%, w/v)	0.1	1.0

$$\text{Coded value} = \frac{\text{actual level} - (\text{high level} + \text{low level})/2}{(\text{high level} - \text{low level})/2} \quad (1)$$

All other formulation and processing variables were kept invariant throughout the study. Encapsulation efficiency (Y_1), particle size (Y_2) and zeta potential (Y_3) were analyzed by using second-order polynomial equation. Polynomial models including interaction and quadratic terms were generated for all the response variables using multiple linear regression analysis (MLRA) approach. The general form of the MLRA model is represented as Eq. (2)

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_1 X_2 + \beta_5 X_1 X_3 + \beta_6 X_2 X_3 + \beta_7 X_1^2 + \beta_8 X_2^2 + \beta_9 X_3^2 \quad (2)$$

Where β_0 is the intercept representing the arithmetic average of all quantitative outcomes of 20 runs β_1 to β_9 are the coefficients computed from the observed experimental values of Y. the term X_1 and X_2 are the coded levels of the independent variables and X_1X_2 represent the interaction and X_i^2 represent the quadratic terms. The interaction terms show how the response changes when two factors were simultaneously changed. The second-degree term (X_i^2) is included to investigate non-linearity. Statistical validity of the polynomials was done on the basis of ANOVA provisions of the MINITAB software. 3-D response surface plots were generated using

the MINITAB software to study the combined effect of different variables on the response. The optimum values of dependent variables to achieve the desired response were calculated using the numerical optimization tool along with desirability approach.

Synthesis of GgR nanoparticles

Table 2: Central composite design illustrating coded values of process variables along with the experimental and predicted values of the responses

Ex. No	X_1	X_2	X_3	Y_1		Y_2		Y_3	
				Exp	Pred	Exp	Pred	Exp	Pred
1	1.00	1.00	0.10	56.50	60.95	694.20	685.49	-36.06	-36.94
2	0.55	5.50	1.31	38.16	34.05	581.20	588.09	-46.31	-44.97
3	1.00	10.00	0.10	81.25	78.99	266.30	242.98	-40.81	-41.96
4	0.10	1.00	1.00	4.98	8.03	728.01	729.12	-5.48	-35.79
5	0.55	5.50	0.55	18.98	16.92	195.50	198.00	-48.20	-47.67
6	0.55	5.50	0.001	72.50	78.14	217.00	263.57	-46.61	-45.19
7	0.55	5.50	0.55	17.25	16.92	235.95	198.00	-48.84	-47.67
8	0.10	10.00	0.10	97.05	94.17	177.35	177.18	-48.65	-48.52
9	0.55	0.001	0.55	10.35	4.73	630.80	674.85	-35.76	-33.50
10	0.55	5.50	0.55	17.15	16.92	187.74	198.00	-47.97	-47.67
11	0.01	5.500	0.55	33.78	42.28	127.70	156.28	-51.16	-50.81
12	0.10	10.00	1.00	51.90	48.24	215.58	202.08	-47.58	-48.16
13	1.00	1.00	1.00	4.01	7.68	948.12	926.07	-37.72	-39.31
14	0.55	13.068	0.55	50.18	52.01	124.38	132.52	-39.09	-38.20
15	0.55	5.50	0.55	26.50	16.92	247.70	198.00	-46.25	-47.67
16	1.31	5.50	0.55	56.50	51.03	364.50	381.35	-52.68	-50.77
17	1.00	10.00	1.00	54.80	60.21	168.35	180.98	-46.24	-46.89
18	0.55	5.50	0.55	15.05	16.92	196.80	198.00	-46.24	-47.67
19	0.10	1.00	0.10	93.08	88.46	436.50	401.65	-37.95	-38.72
20	0.55	5.50	0.55	11.15	16.92	186.64	198.00	-46.28	-47.67

X_1- concentration of gellan gum, X_2- concentration of AOT, X_3- concentration of drug.

Gellan gum nanoparticles were prepared by employing emulsion cross-linking method[29]. To optimize the formulation, gellan gum nanoparticles were prepared based on the central composite design as described in table 2. Briefly, a solution of purified gellan gum and raloxifene HCl were prepared in deionized water (pH 8.0) and methanol respectively. Each one ml of this solution was emulsified into 3 ml of AOT solution in methylene chloride by sonication (Sonics, Vibracell, USA) for 1 minute over ice bath to form a primary emulsion. Secondary w/o/w emulsion was prepared by emulsifying the primary emulsion into 15 ml aqueous solution of polyvinyl alcohol (PVA, 3.5%, w/v) by sonication for 3 minutes over ice bath. Aqueous calcium chloride solution (5 ml, 60%, w/v) was added gradually to the above emulsion with continuous stirring. Methylene chloride was removed under vacuum using a rotary vacuum evaporator (Heidolph, Germany) and the nanoparticles formed were recovered by centrifugation

at 10,000 rpm for 1 hour. Nanoparticles were washed twice with deionized water, centrifuged to remove PVA and unentrapped drug. The pellet so obtained was resuspended in water and lyophilized.

Evaluation of gellan gum nanoparticles

The nanoparticles of gellan gum were evaluated for their particle size, zeta potential, morphology, encapsulation efficiency, and in vitro drug release characteristics.

Zeta potential and average particle size analysis

The stability of the nanoparticles system was evaluated by zeta potential measurements. Zeta potential and average particle size analysis was performed by DLS-Zeta Sizer Nano series (Malvern). The measurement was done at 25°C for 2 minutes.

Scanning electron microscopy

The morphology of the prepared GgR nanoparticles was examined by scanning electron microscope (Hitachi SU6600) with a Schottky field emission electron source operated under low vacuum condition. The sample was kept at a working distance of 10.9 mm from the electron gun and was illuminated with an electron beam operated at an accelerating voltage of 15 kV and 2 µm magnifications.

Encapsulation efficiency

To calculate the actual drug loading, an accurately weighed quantity of nanoparticles was sonicated in 10 ml of methanol for 5 minutes, filtered through 0.45 µ syringe filter. The concentration of raloxifene was estimated using HPLC system (Hitachi, Japan) consisting of a pump (Model L2100), an auto sampler (Model L2200) and an ultraviolet detector (Model L2420). The analytes were detected at 287 nm using a C18 analytic column (Insertsil ODS3: 0.5 µm 15×0, 46 cm^2, GL Sciences Inc, Japan) with acetonitrile and phosphate buffer solution (pH 3.0, 35:65, volume ratio) as mobile phase. Each analysis was performed in triplicate and the flow rate of the mobile phase was 1.0 ml/min at 40°C and Encapsulation efficiency was calculated using the following equation:

%Encapsulation Efficiency = (Actual drug loading/ Theoretical drug loading) × 100 (3)

RESULTS AND DISCUSSION

Gellan gum is an anionic, water soluble heteropolysaccharide which forms insoluble gels on addition of calcium or sodium ions. AOT (Di-octyl sodium sulfosuccinate) is an anionic surfactant containing polar sulphosuccinate head group with large branching Di-octyl side chain. AOT forms reverse micelles in non-polar solvents such as methylene chloride and acquires

bilayer structure in multiple emulsions due to its double chain amphiphilic nature and also forms insoluble salt with cationic calcium ions. In the present study we have used gellan gum -AOT surfactant system to prepare GgR nanoparticles. The aqueous core of gellan gum was entrapped into the reverse micelles formed by the AOT in methylene chloride and further emulsified in the aqueous phase using PVA as a secondary emulsifier. Due to unique properties of the surfactant-polymer system used in this study, the nanoparticles formed are expected to have a calcium crosslinked core composed of gellan and AOT head groups encircled by a hydrophobic matrix made of AOT side chains with the drug encapsulated in the core (Mahesh et al., 2007). Earlier studies on preparation of polymer nanoparticle by emulsion cross link methods showed that the concentration of polymer, surfactant and drug had significant influence on encapsulation, particle size, and zetapotential of the nanoparticles system. Particle size of the nanoparticles influences the drug bioavailability and zetapotential determines the nanoparticles stability. Zetapotential value of more than ±30mV indicates a nanoparticles formulation to be more stable[32] and also affect the nanoparticle intra cellular distribution[33]. So, in this present study, concentrations of gellan gum, AOT and drug were selected as process variables for the optimization of % encapsulation (Y_1), particle size (Y_2) and zetapotential (Y_3) which were taken as response variables. The optimization of process variables was carried out using a central composite design (Table. 2). The results of response generated using the experimental design was fitted into polynomial models and ANOVA test was applied to the polynomial models to estimate the significance of the model. The polynomial models obtained from regression analysis for the response variables Y_1, Y_2 and Y_3 are given in equation 4, 5 and 6 respectively.

$$Y_1 = 119.95-113.5X_1-1.98\ X_2-210.0\ X_3+71.0\ X_1^2+0.185\ X_2^2+102.7\ X_3^2+1.52\ X_1X_2+ 33.5\ X_1X_3 +4.26X_2X_3 \ldots(4)$$

$$Y_2 = 472.3+213.1X_1-84.23X_2-122.9X_3+127.2X_1^2+5.974X_2^2+486.2X_3^2-26.92X_1X_2-107.3X_1X_3- 37.35X_2X_3 \ldots 5)$$

$$Y_3 = -5.41+10.46X_1-4.338X_2 \quad -2.58\ X_3-7.66X_1^2+0.2929X_2^2+6.18X_3^2 \quad +0.591X_1X_2-6.54X_1X_3- 0.317X_2X_3 \ldots(6)$$

The polynomial equations comprise the coefficients for intercept, main effect, interaction terms and higher order effect. The sign and magnitude of the main effects signify the relative influence of each factor on the response. A negative sign denotes antagonistic effect and a positive sign indicates a synergistic effect.

ANOVA test on the quadratic regression model and details of the model summary statistics are presented in table 3 which reveals that the developed response surface model for three responses were significant and adequate without significant 'lack of fit'. It can be observed that all the responses show R^2 value >0.9 which indicates a good correlation between the experimental and predicted responses. The closure the value of R^2 to 1, the better is the correlation between the observed and predicted values. In addition, the predicted R^2 value is in reasonable good agreement with adjusted R^2 value, resulting in reliable models. The factor effects and associated *p*-values for the responsesY_1, Y_2 and Y_3 are presented in table 4. The data showed that significant factors affecting the response Y_1 were the synergistic effects of linear contribution of X_1, X_2 and X_3, quadratic contribution of X_1 and X_3 and interaction effects of X_1 X_3 and X_2X_3. The response Y_2 was significantly affected by the synergistic effects of linear contribution of X_3 and quadratic contributions of X_1, X_2 and X_3 while Y_2 was antagonistically affected by significant factor effects of linear contributions of X_2 and interaction effects of X_1X_2 and X_2X_3. In case of zeta potential (Y_3), antagonistic effect of linear effects of X_1,X_2, quadratic effects of X_1 and interactive effect of X_1X_3 was observed. $Y_3$was Synergistically affected by linear contributions of X_3 and quadratic contribution of X_2 and X_3.

Table 3: Summary of model parameters of the central composite design

Response factor	Model					Lack of fit	
	F-value	Prob>F	R^2	Adj R^2	Pred R^2	F value	Prob>F
Y_1	41.35	<0.0001`	0.9738	0.9503	0.8343	2.55	0.163
Y_2	98.53	<0.0001	0.9888	0.9788	0.9420	2.46	0.172
Y_3	21.77	<0.0001	0.9514	0.9077	0.6799	2.96	0.129

Table 4: Factor effect and *p*-values for responses Y_1, Y_2, and Y_3.

Factor	Y_1		Y_2		Y_3	
	Factor effect	*p*-value	Factor effect	*p*-value	Factor effect	*p*-value
X_1	134.8	0.025	-227	0.426	-30.23	0.042
X_2	20.24	0.003	-268.1	<0.001	-4.890	0.004
X_3	8467	<0.001	46311	<0.001	55.00	0.035
X_1^2	28.74	<0.001	51.5	0.043	-3.100	0.015
X_2^2	0.0749	0.101	2.419	<0.001	0.11863	<0.001
X_3^2	4160	0.000	19691	<0.001	250.4	0.038
X_1X_2	0.616	0.213	-10.90	0.001	0.2392	0.068
X_1X_3	135.8	0.015	-434	0.108	-26.48	0.047
X_2X_3	17.25	0.004	-151.3	<0.000	-1.282	0.298

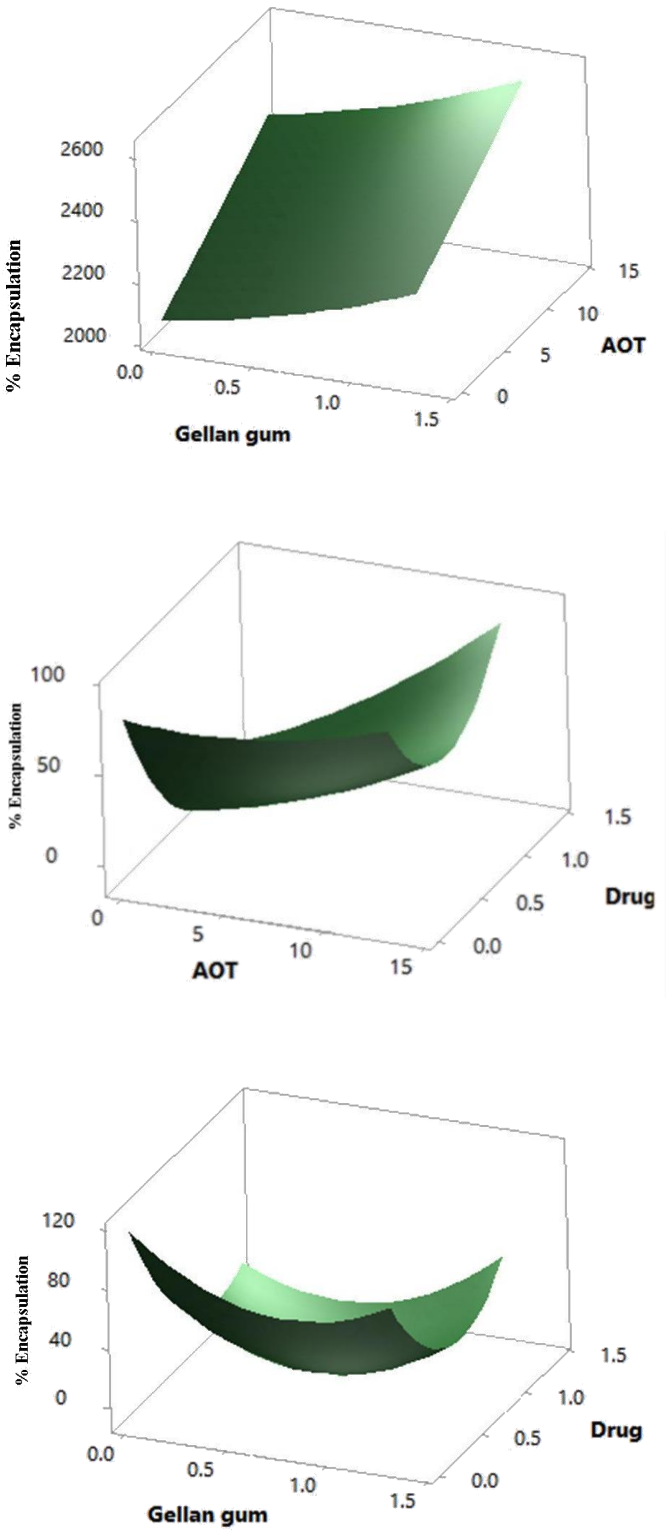

Figure 1. Response surface plots showing combination effect (a) gellan gum and AOT, (b) AOT and drug and (c)gellan gum and drug, on % encapsulation of GgR nanoparticles formulation

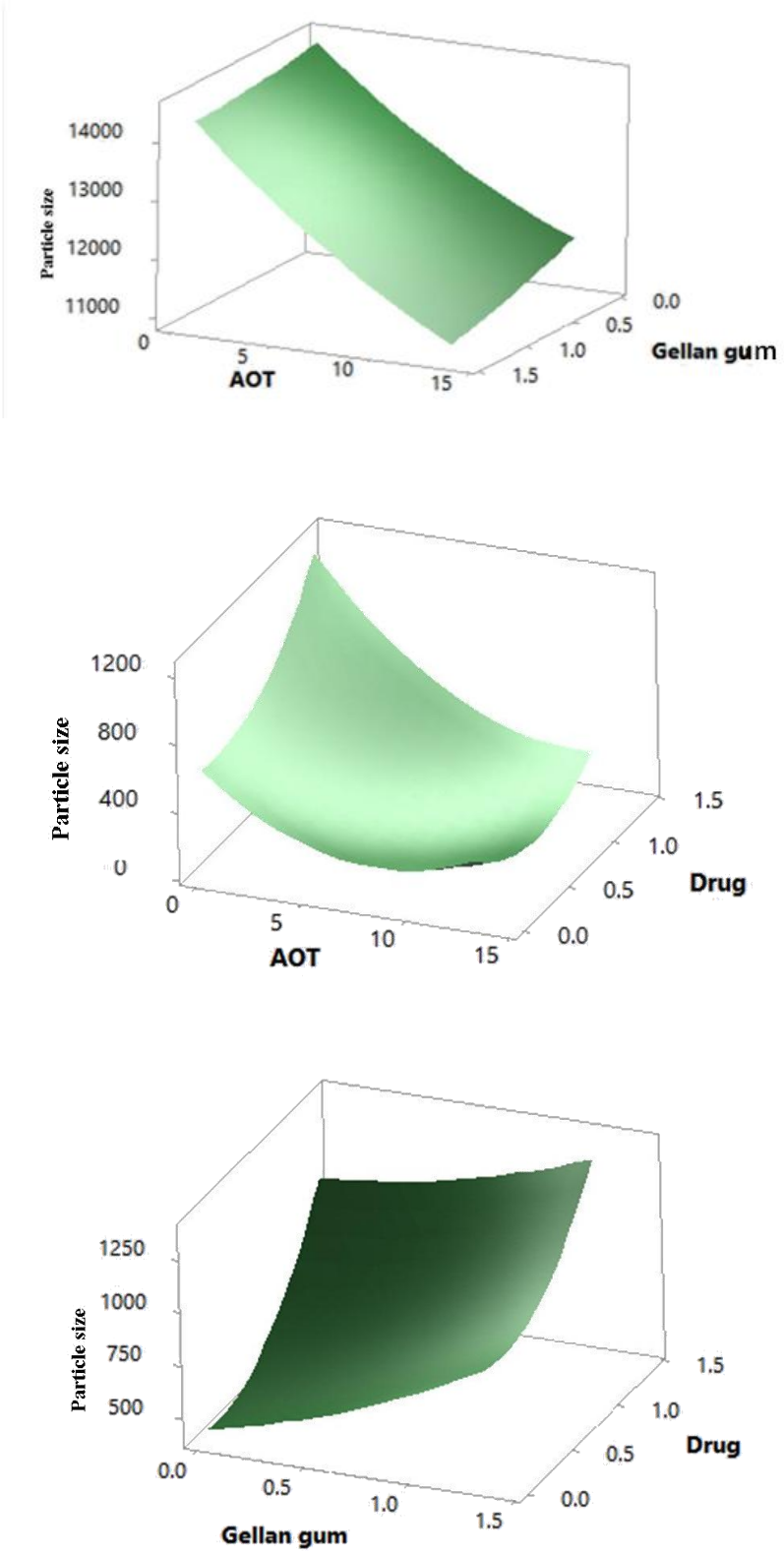

Figure 2. Response surface plots showing combination effect (a) AOT and gellan gum, (b) AOT and drug and (c)gellan gum and drug, on particle size of GgR nanoparticles formulation

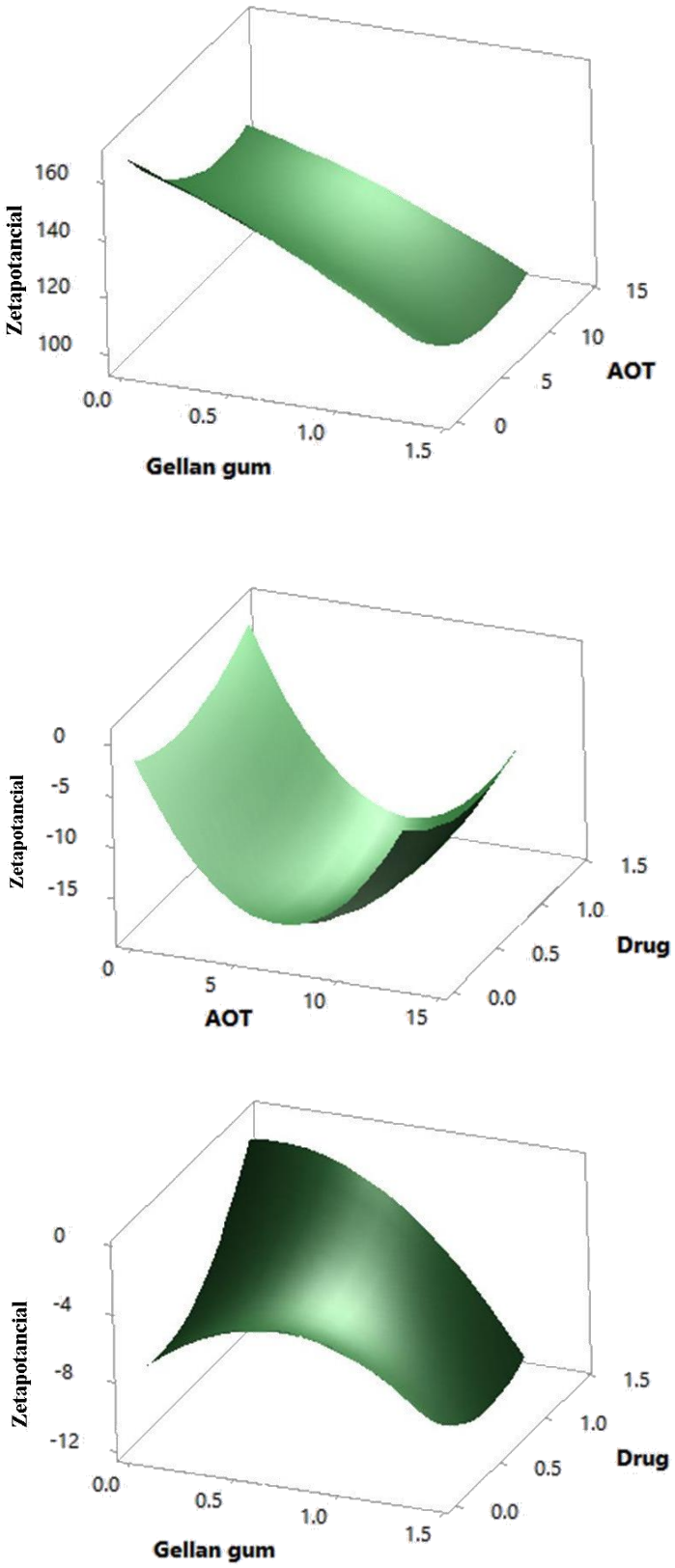

Figure 3. Response surface plots showing combination effect (a) gellan gum and AOT, (b) AOT and drug and (c) gellan gum and drug, on zetapotancial of GgR nanoparticles formulation

The 3-D response surface plots were constructed using the models generated by central composite design (Figures. 1–3). Figure 1 exhibits the combined effect of concentrations of gellan gum/ AOT, AOT /drug and gellan gum/Drug, respectively on % encapsulation of GgR nanoparticles. The plot (Figure. 1a) shows the combined effect of concentration of AOT (X_2) and concentration of drug (X_3) on % encapsulation. It shows that the effect of AOT is more pronounced than the effect of drug concentration. Thus, concentration of AOT is the limiting factor; any smaller variation in AOT concentration will greatly impact the %encapsulation. In this study gellan gum act as a nano-reservoir encapsulated with drug, increasing the amount of AOT allows encapsulation of more drugs within the matrix. The maximum encapsulation of drug within the matrix occurs in nanoparticles prepared using minimum drug concentration and higher AOT concentration. Figure 1b illustrates the combined effect of concentration of AOT and drug, on % encapsulation. It shows that increasing the drug concentration up to a certain level and AOT concentration spontaneously increases the % encapsulation. The plot (Figure. 1c) shows the combined effect of gellan gum and drug on % encapsulation. It shows that % encapsulation will increase while reducing gellan gum and drug concentration from a certain level.

The combined effect of concentrations of AOT/gellan gum, AOT/drug and gellan gum /drug, respectively on particle size of GgR nanoparticles formulation is given in Figure. 2. The plot (Figure. 2a) show an antagonistic curvilinear relationship between the AOT/gellan gum and particle size, with the effect of AOT more pronounced than the gellan gum. AOT, an anionic surfactant forms reverse micelles, increasing the concentration of AOT in the system decreases the particle size. A curvilinear synergistic relationship between the drug and particle size, while a curvilinear antagonistic relationship between the gum and particle size can be observed (Figure. 2b). Increasing the concentration of drug increases the size of the core matrix encapsulating the drug which results in an increase in particle size. Figure 2(c) displays the combined effect of drug concentration and concentration of AOT on particle size, with the effect of AOT more pronounced than the drug concentration.

Figure 3 shows the combined effect of concentrations of gellan gum/AOT, AOT/drug and gellan gum/drug on the zeta potential. It can be observed that AOT, which coats the core comprising of matrix of drug and gum, had the most pronounced effect on zeta potential. AOT being anionic imparts negative charge on the nanoparticles, which increases in magnitude with increasing the concentration of AOT. Desirability approach was applied to develop a new formulation with the optimized responses. This was done with a restricted range for individual responses, %encapsulation (Y_1) in the range of 4–98%, particle size (Y_2) in the range of 125–900 nm, and

the value of zeta potential (Y_3) between −52 to −35 mV as the desired levels to find the optimum levels of process variables in the new formulation. The optimal concentration of process variables were 1.31, 9.87 and 1.08, respectively for gellan gum (%, w/v), AOT (%, w/v) and raloxifene HCl (%, w/v).

CONCLUSION

Raloxifene loaded gellan gum nanoparticles were successfully prepared and the process variables for the preparation of nanoparticles *viz* concentrations of gellan gum , AOT and raloxifene were optimized using a central composite design to get a better formulation with optimum levels of % encapsulation, particle size and zeta potential. Concentration of AOT showed highest impact on % encapsulation, particle size and zeta potential than the concentration of gellan gum and drug. Higher concentration of AOT in the system exhibited lower particle sizes with higher zeta potential and higher % encapsulation. At lower concentration of drug and gellan gum, the nanoparticles system showed higher % encapsulation and smaller particle size. Optimum concentration of gellan gum, AOT and drug were found to be 1.31%, w/v 9.87%, w/v and 1.08%, w/v respectively and at these optimum levels of process variables, the nanoparticles system exhibited 97% encapsulation, 256 nm particle size and -49 mV zeta potential.

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